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(71) Applicant: DUKE UNIVERSITY [US/US]; Erwin Road, Durham, NC 27706 (US).

(72) Inventors: KING, Klim ; 3925 Linden Terrace, Durham, NC 27705 (US). DOHLMAN, Henrik, G. ; 2226 Stewart Street, Berkeley, CA 94705 (US). CARON, Marc, G. ; 2606 Evans Drive, Durham, NC 27705 (US). LEFKOWITZ, Robert, J. ; 3539 Hamstead Court, Durham, NC 27707 (US).

(74) Agents: SIBLEY, Kenneth, D. et al.; Bell, Seltzer, Park & Gibson, P.O. Drawer 34009, Charlotte, NC 28234 (US).

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(54) Title: EXPRESSION OF G PROTEIN COUPLED RECEPTORS IN YEAST

(57) Abstract

Disclosed is a transformed yeast cell containing a first heterologous DNA sequence which codes for a mammalian G protein coupled receptor and a second heterologous DNA sequence which codes for a mammalian G protein α subunit (mammalian G_α). The first and second heterologous DNA sequences are capable of expression in the cell, but the cell is incapable of expressing an endogenous G protein α-subnit (yeast G_α). The cells are useful for screening compounds which affect the rate of dissociation of G_α from $G_{\beta\gamma}$ in a cell. Also disclosed is a novel DNA expression vector useful for making cells as described above. The vector contains a first segment comprising at least a fragment of the extreme amino-terminal coding sequence of a yeast G protein coupled receptor. A second segment is positioned downstream from the first segment (and in correct reading frame therewith), with the second segment comprising a DNA sequence encoding a heterologous G protein coupled receptor.

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EXPRESSION OF G PROTEIN COUPLED RECEPTORS IN YEAST

This invention was made with government support under NIH grant HL16037. The government may have certain rights to this invention.

Field of the Invention

This invention relates to yeast cells expressing heterologous G protein coupled receptors, vectors useful for making such cells, and methods of using the same.

Background of the Invention

The actions of many extracellular signals (for example, neurotransmitters, hormones, odorants, light) are mediated by receptors with seven transmembrane domains (G protein coupled receptors) and heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins). See H. Dohlman, M. Caron, and R. Lefkowitz, Biochemistry 26, 2657 (1987); L. Stryer and H. Bourne, Ann. Rev. Cell Biol. 2, 391 (1986). Such G protein-mediated signaling systems have been identified in organisms as divergent as yeast and man. See H. Dohlman et al., supra; L. Stryer and H. Bourne, supra; K. Blumer and J. Thorner, Annu.Rev. Physiol. (in press). The β2-adrenergic receptor (βAR) is the prototype of the seven-transmembrane-segment class of

ligand binding receptors in mammalian cells. In response to epinephrine or norepinephrine, βAR activates a G protein, G_s, which in turn stimulates adenylate cyclase and cyclic adenosine monophosphate production in the cell. See H. Dohlman et al., supra; L. Stryer and H. Bourne, supra. G protein-coupled pheromone receptors in yeast control a developmental program that culminates in mating (fusion) of a and α haploid cell types to form the a/α diploid. See K. Blumer and J. Thorner, supra; I. Herskowitz, Microbiol. Rev. 52, 536 (1988).

The present invention is based on our continued research into the expression of heterologous G protein coupled receptors in yeast.

Summary of the Invention

A first aspect of the present invention is a transformed yeast cell containing a first heterologous DNA sequence which codes for a mammalian G protein coupled receptor and a second heterologous DNA sequence which codes for a mammalian G protein α subunit (mammalian G_α). The first and second heterologous DNA sequences are capable of expression in the cell, but the cell is incapable of expressing an endogenous G protein α-subunit (yeast G_α). The cell optionally contains a third heterologous DNA sequence, with the third heterologous DNA sequence comprising a pheromone-responsive promotor and an indicator gene positioned downstream from the pheromone-responsive promoter and operatively associated therewith.

A second aspect of the present invention is a method of testing a compound for the ability to affect the rate of dissociation of G_α from $G_{\beta\gamma}$ in a cell. The method comprises: providing a transformed yeast cell as described above; contacting the compound to the cell; and then detecting the rate of dissociation of G_α from $G_{\beta\gamma}$ in the cell. The cells may be provided in an

aqueous solution, and the contacting step carried out by adding the compound to the aqueous solution.

A third aspect of the present invention is a DNA expression vector capable of expressing a transmembrane protein into the cell membrane of yeast cells. The vector contains a first segment comprising at least a fragment of the extreme amino-terminal coding sequence of a yeast G protein coupled receptor. A second segment is positioned downstream from the first segment (and in correct reading frame therewith), with the second segment comprising a DNA sequence encoding a heterologous G protein coupled receptor.

A fourth aspect of the present invention is a yeast cell transformed by a vector as described above.

Brief Description of the Drawings

Figure 1 illustrates the construction of the yeast human β2 Adrenergic Receptor expression plasmid, pYβAR2.

Figure 2 illustrates hβAR ligand binding to membranes from pYβAR2-transformed yeast cells.

Figure 3 shows a comparison of β-adrenergic agonist effects on pheromone-inducible gene activity. α-MF, 10 μM α-mating factor; (-) ISO, 50 μM (-) isoproterenol; (-) ALP, 50 μM (-) alprenolol; (+) ISO, 100 μM (+) isoproterenol.

Detailed Description of the Invention

Nucleotide bases are abbreviated herein as follows:

A=Adenine	G=Guanine
C=Cytosine	T=Thymine

Amino acid residues are abbreviated herein to either three letters or a single letter as follows:

Ala;A=Alanine	Leu;L=Leucine
Arg;R=Arginine	Lys;K=Lysine
Asn;N=Asparagine	Met;M=Methionine
Asp;D=Aspartic acid	Phe;F=Phenylalanine
Cys;C=Cysteine	Pro;P=Proline

Gln;Q=Glutamine	Ser;S=Serine
Glu;E=Glutamic acid	Thr;T=Threonine
Gly;G=Glycine	Trp;W=Tryptophan
His;H=Histidine	Tyr;Y=Tyrosine
Ile;I=Isoleucine	Val;V=Valine

The term "mammalian" as used herein refers to any mammalian species (e.g., human, mouse, rat, and monkey).

The term "heterologous" is used herein with respect to yeast, and hence refers to DNA sequences, proteins, and other materials originating from organisms other than yeast (e.g., mammalian, avian, amphibian), or combinations thereof not naturally found in yeast.

The terms "upstream" and "downstream" are used herein to refer to the direction of transcription and translation, with a sequence being transcribed or translated prior to another sequence being referred to as "upstream" of the latter.

G proteins are comprised of three subunits: a guanyl-nucleotide binding α subunit; a β subunit; and a γ subunit. G proteins cycle between two forms, depending on whether GDP or GTP is bound thereto. When GDP is bound the G protein exists as an inactive heterotrimer, the $G_{\alpha\beta\gamma}$ complex. When GTP is bound the α subunit dissociates, leaving a $G_{\beta\gamma}$ complex. Importantly, when a $G_{\alpha\beta\gamma}$ complex operatively associates with an activated G protein coupled receptor in a cell membrane, the rate of exchange of GTP for bound GDP is increased and, hence, the rate of dissociation of the bound α subunit from the $G_{\beta\gamma}$ complex increases. This fundamental scheme of events forms the basis for a multiplicity of different cell signaling phenomena. See generally Stryer and Bourne, supra.

Any mammalian G protein coupled receptor, and the DNA sequences encoding these receptors, may be employed in practicing the present invention. Examples

of such receptors include, but are not limited to, dopamine receptors, muscarinic cholinergic receptors, α-adrenergic receptors, β-adrenergic receptors, opiate receptors, cannabinoid receptors, and serotonin receptors. The term receptor as used herein is intended to encompass subtypes of the named receptors, and mutants and homologs thereof, along with the DNA sequences encoding the same.

The human D_1 dopamine receptor cDNA is reported in A. Dearry et al., Nature 347, 72-76 (1990).

The rat D_2 dopamine receptor cDNA is reported in J. Bunzow et al., Nature 336, 783-787 (1988); see also O. Civelli, et al., PCT Appln. WO 90/05780 (all references cited herein are to be incorporated herein by reference).

Muscarinic cholinergic receptors (various subtypes) are disclosed in E. Peralta et al., Nature 343, 434 (1988) and K. Fukuda et al., Nature 327, 623 (1987).

Various subtypes of α_2-adrenergic receptors are disclosed in J. Regan et al., Proc. Natl. Acad. Sci. USA 85, 6301 (1988) and in R. Lefkowitz and M. Caron, J. Biol. Chem. 263, 4993 (1988).

Serotonin receptors (various subtypes) are disclosed in S. Peroutka, Ann. Rev. Neurosci. 11, 45 (1988).

A cannabinoid receptor is disclosed in L. Matsuda et al., Nature 346, 561 (1990).

Any DNA sequence which codes for a mammalian G α subunit (G_α) may be used to practice the present invention. Examples of mammalian G α subunits include G_s α subunits, G_i α subunits, G_o α subunits, G_z α subunits, and transducin α subunits. See generally Stryer and Bourne, supra. G proteins and subunits useful for practicing the present invention include subtypes, and mutants and homologs thereof, along with the DNA sequences encoding the same.

Heterologous DNA sequences are expressed in a host by means of an expression vector. An expression vector is a replicable DNA construct in which a DNA sequence encoding the heterologous DNA sequence is operably linked to suitable control sequences capable of effecting the expression of a protein or protein subunit coded for by the heterologous DNA sequence in the intended host. Generally, control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and (optionally) sequences which control the termination of transcription and translation.

Vectors useful for practicing the present invention include plasmids, viruses (including phage), and integratable DNA fragments (i.e., fragments integratable into the host genome by homologous recombination). The vector may replicate and function independently of the host genome, as in the case of a plasmid, or may integrate into the genome itself, as in the case of an integratable DNA fragment. Suitable vectors will contain replicon and control sequences which are derived from species compatible with the intended expression host. For example, a promoter operable in a host cell is one which binds the RNA polymerase of that cell, and a ribosomal binding site operable in a host cell is one which binds the endogenous ribosomes of that cell.

DNA regions are operably associated when they are functionally related to each other. For example: a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous and, in the case of leader sequences, contiguous and in reading phase.

Transformed host cells of the present invention are cells which have been transformed or transfected with the vectors constructed using recombinant DNA techniques and express the protein or protein subunit coded for by the heterologous DNA sequences. In general, the host cells are incapable of expressing an endogenous G protein α-subunit (yeast G_α). The host cells do, however, express a complex of the G protein β subunit and the G protein γ subunit ($G_{\beta\gamma}$). The host cells may express endogenous $G_{\beta\gamma}$, or may optionally be engineered to express heterologous $G_{\beta\gamma}$ (e.g., mammalian) in the same manner as they are engineered to express heterologous G_α.

A variety of yeast cultures, and suitable expression vectors for transforming yeast cells, are known. See, e.g., U.S. Patent No. 4,745,057; U.S. Patent No. 4,797,359; U.S. Patent No. 4,615,974; U.S. Patent No. 4,880,734; U.S. Patent No. 4,711,844; and U.S. Patent No. 4,865,989. Saccharomyces cerevisiae is the most commonly used among the yeast, although a number of other strains are commonly available. See, e.g., U.S. Patent No. 4,806,472 (Kluveromyces lactis and expression vectors therefor); 4,855,231 (Pichia pastoris and expression vectors therefor). Yeast vectors may contain an origin of replication from the 2 micron yeast plasmid or an autonomously replicating sequence (ARS), a promoter, DNA encoding the heterologous DNA sequences, sequences for polyadenylation and transcription termination, and a selection gene. An exemplary plasmid is YRp7, (Stinchcomb et al., Nature 282, 39 (1979); Kingsman et al., Gene 7, 141 (1979); Tschemper et al., Gene 10, 157 (1980)). This plasmid contains the TRP1 gene, which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, Genetics 85, 12 (1977)). The presence of the TRP1 lesion in the

yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem. 255, 2073 (1980) or other glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg. 7, 149 (1968); and Holland et al., Biochemistry 17, 4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., EPO Publn. No. 73,657. Other promoters, which have the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned metallothionein and glyceraldehyde-3-phosphate dehydrogenase, as well as enzymes responsible for maltose and galactose utilization.

In constructing suitable expression plasmids, the termination sequences associated with these genes may also be ligated into the expression vector 3' of the heterologous coding sequences to provide polyadenylation and termination of the mRNA.

A novel DNA expression vector described herein which is particularly useful for carrying out the present invention contains a first segment comprising at least a fragment of the extreme amino-terminal coding sequence of a yeast G protein coupled receptor and a second segment downstream from said

first segment and in correct reading frame therewith, the second segment comprising a DNA sequence encoding a heterologous G protein coupled receptor (e.g., a mammalian G protein coupled receptor). In a preferred embodiment, this vector comprises a plasmid. In constructing such a vector, a fragment of the extreme amino-terminal coding sequence of the heterologous G protein coupled receptor may be deleted. The first and second segments are operatively associated with a promoter, such as the GAL1 promoter, which is operative in a yeast cell. Coding sequences for yeast G protein coupled receptors which may be used in constructing such vectors are exemplified by the gene sequences encoding yeast pheromone receptors (e.g., the STE2 gene, which encodes the α-factor receptor, and the STE3 gene, which encodes the a-factor receptor). The levels of expression obtained from these novel vectors are enhanced if at least a fragment of the 5′-untranslated region of a yeast G protein coupled receptor gene (e.g., a yeast pheromone receptor gene; see above) is positioned upstream from the first segment and operatively associated therewith.

Any of a variety of means for detecting the dissociation of G_α from $G_{\beta\gamma}$ can be used in connection with the present invention. The cells could be disrupted and the proportion of these subunits and complexes determined physically (i.e., by chromatography). The cells could be disrupted and the quantity of G_α present assayed directly by assaying for the enzymatic activity possessed by G_α in isolation (i.e., the ability to hydrolyze GTP to GDP). Since whether GTP or GDP is bound to the G protein depends on whether the G protein exists as a $G_{\beta\gamma}$ or $G_{\alpha\beta\gamma}$ complex, dissociation can be probed with radiolabelled GTP. As explained below, morphological changes in the cells can be observed. A particularly convenient method, however, is to provide in the cell a third heterologous

DNA sequence, wherein the third heterologous DNA sequence comprises a pheromone-responsive promotor and an indicator gene positioned downstream from the pheromone-responsive promotor and operatively associated therewith. This sequence can be inserted with a vector, as described in detail herein. With such a sequence in place, the detecting step can be carried out by monitoring the expression of the indicator gene in the cell. Any of a variety of pheromone responsive promoters could be used, examples being the BAR1 gene promoter and the FUS1 gene promoter. Likewise, any of a broad variety of indicator genes could be used, with examples including the HIS3 gene and the LacZ gene.

As noted above, transformed host cells of the present invention express the protein or protein subunit coded for by the heterologous DNA sequence. When expressed, the G protein coupled receptor is located in the host cell membrane (i.e., physically positioned therein in proper orientation for both the stereospecific binding of ligands on the extracellular side of the cell membrane and for functional interaction with G proteins on the cytoplasmic side of the cell membrane).

The ability to control the yeast pheromone response pathway by expression of a heterologous adrenergic receptor and its cognate G protein α-subunit has the potential to facilitate structural and functional characterization of mammalian G protein-coupled receptors. By scoring for responses such as growth arrest or β-galactosidase induction, the functional properties of mutant receptors can now be rapidly tested. Similarly, as additional genes for putative G protein-coupled receptors are isolated, numerous ligands can be screened to identify those with activity toward previously unidentified receptors. See F. Libert et al., Science 244, 569 (1989); M. S. Chee

et al., Nature 344, 774 (1990). Moreover, as additional genes coding for putative G protein α-subunits are isolated, they can be expressed in cells of the present invention and screened with a variety of G protein coupled receptors and ligands to characterize these subunits. These cells can also be used to screen for compounds which affect receptor-G protein interactions.

Cells of the present invention can be deposited in the wells of microtiter plates in known, predetermined quantities to provide standardized kits useful for screening compounds in accordance with the various screening procedures described above.

The following Examples are provided to further illustrate various aspects of the present invention. They are not to be construed as limiting the invention.

EXAMPLE 1

Construction of the Human β2-Adrenergic Expression Vector pYβAR2 and Expression in Yeast

To attain high level expression of the human β2-adrenergic receptor (hβAR) in yeast, a modified hβAR gene was placed under the control of the GAL1 promoter in the multicopy vector, YEp24 (pYβAR2).

Figure 1 illustrates the construction of yeast expression plasmid pYβAR2. In pYβAR2, expression of the hβAR sequence is under the control of the GAL1 promoter. Figure 1A shows the 5′-untranslated region and the first 63 basepairs (bp) of coding sequence of the hβAR gene in pTZNAR, B. O'Dowd et al., J. biol. Chem. 263, 15985 (1988), which was removed by Aat II cleavage and replaced with a synthetic oligonucleotide corresponding to 11 bp of noncoding and 42 bp of coding sequence from the STE2 gene (SEQ ID NO:1; SEQ ID NO:2). See N. Nakayama et al., EMBO J. 4, 2643 (1985); A. Burkholder and L. Hartwell, Nucleic Acids Res. 13, 8463

(1985). The resulting plasmid, pTZYNAR, contains the modified hβAR gene flanked by Hind III sites in noncoding sequences with the 3′ Hind III site given as SEQ ID NO:3 herein. The Hind III-Hind III fragment was isolated from pTZYNAR and inserted into pAAH5 such that the 3′- untranslated sequence of the modified hβAR gene was followed by 450 bp containing termination sequences from the yeast ADH1 gene. See G. Ammerer, Methods. Enzymol. 101, 192 (1983).

As illustrated in Figure 1B, pyβAR2 was constructed by inserting the Bam HI - Bam HI fragment containing hβAR and ADH1 sequences into YEpG24. E. Wyckoff and T. Hsieh, Proc. Natl. Acad. Sci. U.S.A. 85, 6272 (1988). Where maximum expression was sought, cells were cotransformed with plasmid pMTL9 (from Dr. S. Johnston) containing LAC9, a homolog of the S. cerevisiae GAL4 transactivator protein required for GAL1-regulated transcription. J. Salmeron et al., Mol. Cell. Biol. 9, 2950 (1989). Cells grown to late exponential phase were induced in medium containing 3% galactose, supplemented with about 10 μM alprenolol, and grown for an additional 36 hours. Standard methods for the maintenance of cells were used. See F. Sherman et al., Methods in Yeast Genetics (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986).

Maximal expression required (i) expression of a transcriptional transactivator protein (LAC9), (ii) replacement of the 5′ untranslated and extreme NH_2-terminal coding sequence of the hβAR gene with the corresponding region of the yeast STE2 (α-factor receptor) gene, (iii) induction with galactose when cell growth reached late exponential phase, and, (iv) inclusion of an adrenergic ligand in the growth medium during induction.

The plasmid pYβAR2 was deposited in accordance with the provisions of the Budapest Treaty at the American Type Culture Collection, 12301 Parklawn

defined as the amount of total binding (circles) minus nonspecific binding measured in the presence of 10 μM (-) alprenolol (squares). A K_d of 93 pM for ^{125}I-CYP binding was obtained and used to calculate agonist affinities (below). (B) Displacement of 18 pM ^{125}I-CYP with various concentrations of agonists was used to determine apparent low affinity K_i values (non G protein coupled, determined in the presence of 50 μM GTP) for receptor binding, squares; (-) isoproterenol, circles; (-) epinephrine, downward-pointing triangles; (+) isoproterenol, upward pointing triangles; (-) norepinephrine.

COMPARATIVE EXAMPLE A

Ligand Binding Affinity for hβAR Expressed in Yeast and Mammalian Cells

The binding data of Figures 2 (A) and (B) were analyzed by nonlinear least squares regression, see A. DeLean et al., Mol. Pharmacol. 21, (1982), and are presented in Table I. Values given are averages of measurements in triplicate, and are representative of 2 - 3 experiments. Binding affinities in yeast were nearly identical to those observed previously for hβAR expressed in mammalian cells.

Drive, Rockville, MD 20852 USA, on September 11, 1990, and has been assigned ATCC Accession No. 40891.

EXAMPLE 2

Binding Affinity of hβAR Ligands in Yeast Transformed with pYβAR2

A primary function of cell surface receptors is to recognize only appropriate ligands among other extracellular stimuli. Accordingly, ligand binding affinities were determined to establish the functional integrity of hβAR expressed in yeast. As discussed in detail below, an antagonist, ^{125}I-labeled cyanopindolol (^{125}I-CYP), bound in a saturable manner and with high affinity to membranes prepared from pYβAR2-transformed yeast cells. By displacement of ^{125}I-CYP with a series of agonists, the order of potency and stereospecificity expected for hβAR was observed.

SC261 cells (MATa ura3-52 trp1 leu2 prb1-1122 pep4-3 prc1-407) (from Dr. S. Johnston) harboring pYβAR2 (URA3) and pMTL9 (LEU2) were grown in minimal glucose-free selective media to late log phase (OD_{600} = 5.0), and then induced with the addition of 3% galactose and 40 μM alprenolol. After 36 hours, cells were harvested and spheroplasts were prepared as described. See E. Wyckoff and T. Hsieh, Proc. Natl. Acad. Sci. U.S.A. 85, 6272 (1988). Briefly, the spheroplasts were resuspended in 50 mM Tris-HCl pH 7.4, 5 mM EDTA and were lysed by vortex mixing with glass beads for three one-min periods at 4°C. Crude membranes were prepared from the lysates and binding assays with ^{125}I-CYP were performed by methods described previously. See H. Dohlman et al., Biochemistry 29, 2335 (1990).

Figure 2 illustrates hβAR ligand binding to membranes from pYβAR2-transformed yeast cells. (A) B_{max} (maximum ligand bound) and K_d (ligand dissociation constant) values were determined by varying ^{125}I-CYP concentrations (5 - 400 pM). Specific binding was

Table 1

Comparison of ligand Binding Parameters for High Level Expression of Human β-Adrenergic Receptor in Yeast and COS-7 Cells*

	Yeast SC261 (pYβAR2, pMTL9)	Monkey COS-7 (pBC12:hβAR)
^{125}I-CYP:		
$^{1}K_d$	0.093 nM ± 0.013	0.110 nM ±0.009
$^{2}B_{max}$	115 pmol/mg	24 pmol/mg
$^{3}K_i$(M):		
(-) isoproterenol	103 ± 26	130 ± 15
(+) isoproterenol	3670 ± 420	4000 ± 184
(-) epinephrine	664 ± 123	360 ± 30
(-) norepinephrine	6000 ± 1383	5800 ± 373

*Values derived from Fig. 2 and H. Dohlman et al., Biochemistry 29, 2335 (1990).; ± S.E.
$^{1}K_d$, ligand dissociation constant
$^{2}B_{max}$, maximum ligand bound
$^{3}K_i$, inhibition constant

EXAMPLE 3

Agonist-Dependent Activation of Mating Signal Transduction in Yeast Expressing hβAR

A second major function of a receptor is agonist-dependent regulation of downstream components in the signal transduction pathway. Because the pheromone-responsive effector in yeast is not known, indirect biological assays are the most useful indicators of receptor functionality. See K. Blumer and J. Thorner, Annu.Rev. Physiol. in press; I. Herskowitz, Microbiol. Rev. 52, 536 (1988). In yeast

cells expressing high concentrations of hβAR, no agonist-dependent activation of the mating signal transduction pathway could be detected by any of the typical in vivo assays; for example, imposition of G1 arrest, induction of gene expression, alteration of morphology (so-called "shmoo" formation) or stimulation of mating. A likely explanation for the absence of responsiveness is that hβAR was unable to couple with the endogenous yeast G protein.

EXAMPLE 4

Coexpression of hβAR and Mammalian G_s α-Subunit in Yeast

Expression of a mammalian G_s α-subunit can correct the growth defect in yeast cells lacking the corresponding endogenous protein encoded by the GPA1 gene. See C. Dietzel and J. Kurjan, Cell 50, 1001 (1987). Moreover, specificity of receptor coupling in mammalian cells is conferred by the α-subunit of G proteins. See L. Stryer and H. Bourne, Annu. Rev. Cell Biol. 2, 391 (1988). Thus, coexpression of hβAR and a mammalian G_s α-subunit ($G_s\alpha$) in yeast was attempted to render the yeast responsive to adrenergic ligands. Accordingly, a cDNA encoding rat $G_s\alpha$ under the control of the copper-inducible CUP1 promoter was introduced on a second plasmid, pYSK136Gαs. See C. Dietzel and J. Kurjan, Cell 50, 1001 (1987). In yeast (NNY19) coexpressing hβAR and rat $G_s\alpha$, but containing wild-type GPA1, no adrenergic agonist-induced shmoo formation, a characteristic morphological change of yeast in response to mating pheromone, was observed.

EXAMPLE 5

Coexpression of hβAR and Mammalian $G_s\alpha$-Subunit in Yeast Lacking an Endogenous G Protein α-Subunit

To prevent interference by the endogenous yeast G protein α-subunit, gpa1 mutant cells (strain

8c) were used.

Yeast strain 8c (MATa ura3 leu2 his3 trp1 gpa1::HIS3), I. Miyajima et al., Cell 50, 1011 (1987), carrying plasmids pYSK136Gαs (TRP1), C. Dietzel and J. Kurjan, Cell 50, 1001 (1987), pMTL9 (LEU2), J. Salmeron et al., Mol. Cell. Biol. 9, 2950 (1989), and pYβAR2 (URA3) was maintained on glucose-free minimal selective plates containing 3% glycerol, 2% lactic acid, 50 μM $CuSO_4$ and 3% galactose. Colonies were transferred to similar plates containing 0.5 mM ascorbic acid and the indicated adrenergic ligand(s). After 16-20 hours at 30°C, the colonies were transferred to similar liquid media at a density of 10^6-10^7 cells/ml and examined by phase contrast microscopy.

Morphologies of yeast cells cotransformed with pYβAR2, pMTL9, and pYSK136Gαs were examined after incubation with (A) no adrenergic agent; (B) 100 μM (-) isoproterenol; (C) 100 μM (-) isoproterenol and 50 μM (-) alprenolol; and (D) 100 μM (+) isoproterenol. Results showed that treatment of 8c cells coexpressing hβAR and rat $G_s\alpha$ with the β-adrenergic agonist isoproterenol indeed induced shmoo formation, and that this effect was blocked by the specific antagonist alprenolol.

EXAMPLE 6

Coexpression of hβAR and Mammalian $G_s\alpha$-Subunit in Yeast Containing a β-Galactosidase Signal Sequence

The isoproterenol-induced morphological response of 8c cells coexpressing hβAR and rat $G_s\alpha$ suggested that these components can couple to each other and to downstream components of the pheromone response pathway in yeast lacking the endogenous G α-subunit. To confirm that the pheromone signaling pathway was activated by hβAR and rat $G_s\alpha$, agonist induction of the pheromone-responsive FUS1 gene promoter was measured in a strain of yeast derived from

8c cells (8c1) in which a FUS1-lacZ gene fusion had been stably integrated into the genome. See S. Nomoto et al., EMBO J. 9, 691 (1990).

Strains 8c (Fig. 3, legend) and NNY19 (MATa ura3 leu2 his3 trp1 lys2 FUS1-LacZ::LEU2) were modified by integrative transformation with YIpFUS102 (LEU2), S. Nomoto et al., supra, and designated 8c1 and NNY19, respectively. These strains were transformed with pYβAR2 and pYSK136Gαs and maintained on minimal selective plates containing glucose and 50 μM $CuSO_4$. Colonies were inoculated into minimal selective media (3% glycerol, 2% lactic acid, 50 μM $CuSO_4$), grown to early log phase (OD_{600} = 1.0), and induced for 12 hours by addition of 3% galactose. Cells were washed and resuspended in induction media (OD_{600} = 5.0) containing 0.5 mM ascorbic acid and the indicated ligands. After a 4 hour incubation at 30°C, cells were harvested, resuspended into 1 ml of Z-buffer, see J. Miller, Experiments in Molecular Genetics (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972), supplemented with 0.0075% SDS, and β-galactosidase activities were determined in 3 - 4 independent experiments as described previously. See J. Miller, supra.

Figure 3 shows a comparison of β-adrenergic agonist effects on pheromone-inducible gene activity. α-MF, 10 μM α-mating factor; (-) ISO, 50 μM (-) isoproterenol; (-) ALP, 50 μM (-) alprenolol; (+) ISO, 100 μM (+) isoproterenol. In 8c1 (gpa1) cells coexpressing hβAR and rat $G_s\alpha$, a dramatic isoproterenol-stimulated induction of β-galactosidase activity was observed. Agonist stimulation was stereoselective and was blocked by addition of a specific antagonist. Agonist responsiveness was dependent on expression of both hβAR and rat $G_s\alpha$, and required a strain in which the endogenous G protein α-subunit was disrupted. The final β-galactosidase activity achieved in response to isoproterenol in transformed 8c1 cells was comparable

to that induced by α-factor in nontransformed cells that express GPA1 (NNY19), although basal β-galactosidase activity in NNY19 cells was considerably lower than in 8c1 cells. Taken together, our results indicated that coexpression of hβAR and rat $G_s\alpha$ was sufficient to place under catecholamine control key aspects of the mating signal transduction pathway in yeast. However, the adrenergic agonist did not stimulate mating in either 8c cells or NNY19 cells coexpressing hβAR and rat $G_s\alpha$, in agreement with recent observations that yeast pheromone receptors, in addition to binding pheromones, participate in other recognition events required for mating. See A. Bender and G. Sprague, Genetics 121, 463 (1989).

hβAR stimulates adenylate cyclase in animal cells via the action of the α-subunit of its G protein. In contrast, mating factor receptors in yeast trigger their effector via the action of the βγ subunits. M. Whiteway et al., Cell 56, 476 (1989). Our present results indicate that activation of hβAR in yeast leads to dissociation of mammalian $G_s\alpha$ from yeast βγ, and it is the βγ subunits that presumably elicit the response.

The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: King, Klim
Dohlman, Henrik G.
Caron, Mark G.
Lefkowitz, Robert J.

(ii) TITLE OF INVENTION: Expression of G Protein Coupled Receptors in Yeast

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Kenneth D. Sibley; Bell, Seltzer, Park and Gibson
(B) STREET: Post Office Drawer 34009
(C) CITY: Charlotte
(D) STATE: North Carolina
(E) COUNTRY: U.S.A.
(F) ZIP: 28234

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US91/06605
(B) FILING DATE: 12-SEP-1991
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/581714
(B) FILING DATE: 13-SEP-1990

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Sibley, Kenneth D.
(B) REGISTRATION NUMBER: 31,665
(C) REFERENCE/DOCKET NUMBER: 5405-17-1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 919-881-3140
(B) TELEFAX: 919-881-3175
(C) TELEX: 575102

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 80 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 30..80

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GAATTCAACG TTGGATCCAA GAATCAAAA ATG TCT GAT GCG GCT CCT TCA TTG        53
                                Met Ser Asp Ala Ala Pro Ser Leu
                                  1               5

AGC AAT CTA TTT TAT GAC GTC ACG CAG                                  80
Ser Asn Leu Phe Tyr Asp Val Thr Gln
     10                  15
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Ser Asp Ala Ala Pro Ser Leu Ser Asn Leu Phe Tyr Asp Val Thr
  1               5                  10                  15

Gln
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
AACGTT                                                               6
```

THAT WHICH IS CLAIMED IS:

1. A transformed yeast cell containing a first heterologous DNA sequence which codes for a mammalian G protein coupled receptor and a second heterologous DNA sequence which codes for a mammalian G protein α subunit (mammalian G_α), wherein said first and second heterologous DNA sequences are capable of expression in said cell, and wherein said cell is incapable of expressing an endogenous G protein α-subunit (yeast G_α).

2. A transformed yeast cell according to claim 1, wherein said first heterologous DNA sequence is carried by a plasmid.

3. A transformed yeast cell according to claim 1, wherein said second heterologous DNA sequence is carried by a plasmid.

4. A transformed yeast cell according to claim 1, wherein said mammalian G protein α subunit is selected from the group consisting of G_s α subunits, G_i α subunits, G_o α subunits, G_z α subunits, and transducin α subunits.

5. A transformed yeast cell according to claim 1 which expresses a complex of the G protein β subunit and the G protein γ subunit ($G_{\beta\gamma}$).

6. A transformed yeast cell according to claim 5 which expresses endogenous $G_{\beta\gamma}$.

7. A transformed yeast cell according to claim 1, wherein said first heterologous DNA sequence codes for a mammalian G protein-coupled receptor selected from the group consisting of dopamine receptors, muscarinic cholinergic receptors, α-adrenergic receptors, β-adrenergic receptors, opiate receptors, cannabinoid receptors, and serotonin receptors.

8. A transformed yeast cell according to claim 1 further comprising a third heterologous DNA sequence, wherein said third heterologous DNA sequence comprises a pheromone-responsive promotor and an indicator gene positioned downstream from said pheromone-responsive promoter and operatively associated therewith.

9. A transformed yeast cell according to claim 8, wherein said pheromone responsive promoter is selected from the group consisting of the BAR1 gene promoter and the FUS1 gene promoter, and wherein said indicator gene is selected from the group consisting of the HIS3 gene and the LacZ gene.

10. A method of testing a compound for the ability to affect the rate of dissociation of G_α from $G_{\beta\gamma}$ in a cell, comprising:

providing a transformed yeast cell containing a first heterologous DNA sequence which codes for a mammalian G protein coupled receptor and a second heterologous DNA sequence which codes for a mammalian G_α, wherein said first and second heterologous DNA sequences are capable of expression in said cell, wherein said cell is incapable of expressing endogenous G_α, and wherein said cell expresses $G_{\beta\gamma}$;

contacting said compound to said cell; and

detecting the rate of dissociation of G_α from $G_{\beta\gamma}$ in said cell.

11. A method according to claim 10, wherein said yeast cells are provided in an aqueous solution and said contacting step is carried out by adding said compound to said aqueous solution.

12. A method according to claim 10, wherein said mammalian G protein α subunit is selected from the group consisting of G_s α subunits, G_i α subunits, G_o α subunits, G_z α subunits, and transducin α subunits.

13. A method according to claim 10, wherein said yeast cell expresses endogenous $G_{\beta\gamma}$.

14. A method according to claim 10, wherein said first heterologous DNA sequence codes for a mammalian G protein-coupled receptor selected from the group consisting of dopamine receptors, muscarinic cholinergic receptors, α-adrenergic receptors, β-adrenergic receptors, opiate receptors, cannabinoid receptors, and serotonin receptors.

15. A method according to claim 10, said yeast cell further comprising a third heterologous DNA sequence, wherein said third heterologous DNA sequence comprises a pheromone-responsive promotor and an indicator gene positioned downstream from said pheromone-responsive promoter and operatively associated therewith;

and wherein said detecting step is carried out by monitoring the expression of said indicator gene in said cell.

16. A DNA expression vector capable of expressing a transmembrane protein into the cell membrane of yeast cells, comprising:

a first segment comprising at least a fragment of the extreme amino-terminal coding sequence of a yeast G protein coupled receptor; and

a second segment downstream from said first segment and in correct reading frame therewith, said second segment comprising a DNA sequence encoding a heterologous G protein coupled receptor.

17. A DNA expression vector according to claim 16, wherein a fragment of the extreme amino-terminal coding sequence of said heterologous G protein coupled receptor is absent.

18. A DNA expression vector according to claim 16, wherein said first and second segments are operatively associated with a promoter operative in a yeast cell.

19. A DNA expression vector according to claim 18, wherein said promoter is the GAL1 promoter.

20. A DNA expression vector according to claim 16, wherein said first segment comprises at least a fragment of the extreme amino-terminal coding sequence of a yeast pheromone receptor.

21. A DNA expression vector according to claim 16, wherein said first segment comprises at least a fragment of the extreme amino-terminal coding sequence of a yeast pheromone receptor selected from the group consisting of the STE2 gene and the STE3 gene.

22. A DNA expression vector according to claim 16, further comprising at least a fragment of the 5'-untranslated region of a yeast G protein coupled receptor gene positioned upstream from said first segment and operatively associated therewith.

23. A DNA expression vector according to claim 16, further comprising at least a fragment of the 5'-untranslated region of a yeast pheromone receptor gene positioned upstream from said first segment and operatively associated therewith.

24. A DNA expression vector according to claim 23, wherein said yeast pheromone receptor gene is selected from the group consisting of the STE2 gene and the STE3 gene.

25. A DNA expression vector according to claim 16, said vector comprising a plasmid.

26. A DNA expression vector according to claim 16, said second segment comprising a DNA sequence encoding a mammalian G protein coupled receptor.

27. A DNA expression vector according to claim 16, said second segment comprising a DNA sequence encoding a mammalian G protein-coupled receptor selected from the group consisting of dopamine receptors, muscarinic cholinergic receptors, α-adrenergic receptors, β-adrenergic receptors, opiate receptors, cannabinoid receptors, and serotonin receptors.

28. A yeast cell carrying a DNA expression vector according to claim 16.

FIG. 1A.

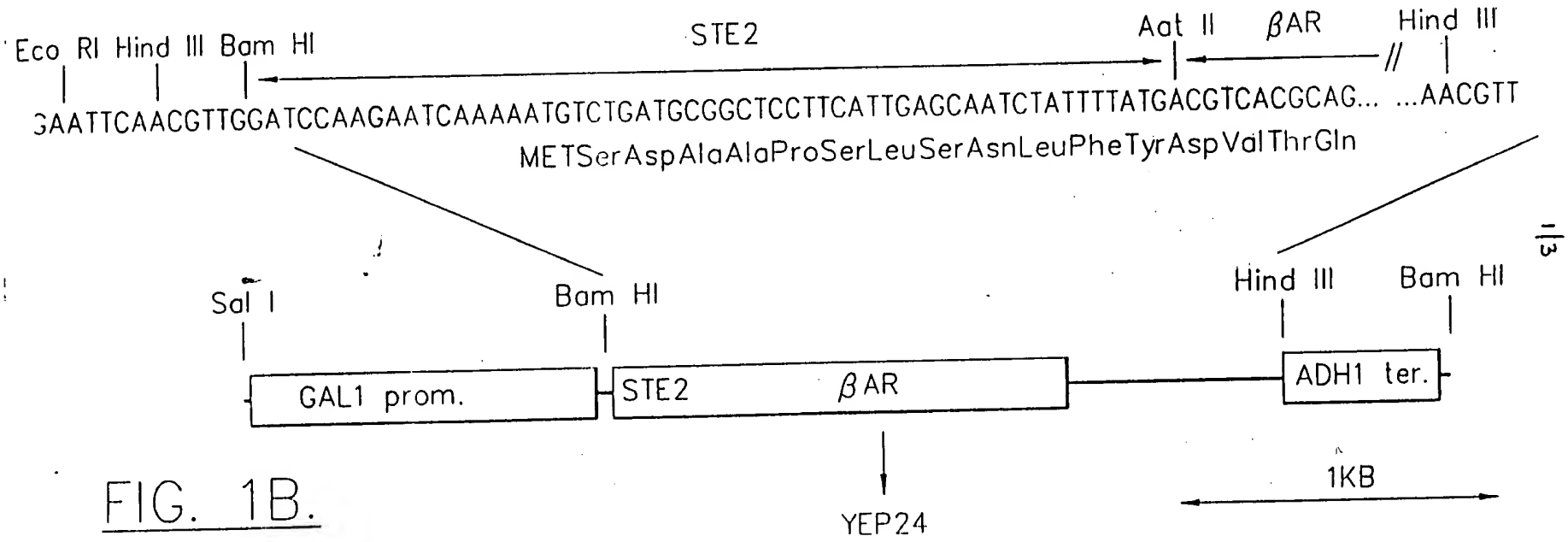
Eco RI Hind III Bam HI
STE2
Aat II
βAR
Hind III
GAATTCAACGTTGGATCCAAGAATCAAAAATGTCTGATGCGGCTCCTTCATTGAGCAATCTATTTTATGACGTCACGCAG... ...AACGTT
METSerAspAlaAlaProSerLeuSerAsnLeuPheTyrAspValThrGln
Sal I
Bam HI
Hind III
Bam HI
GAL1 prom.
STE2
βAR
ADH1 ter.
YEP24
1KB

FIG. 1B.

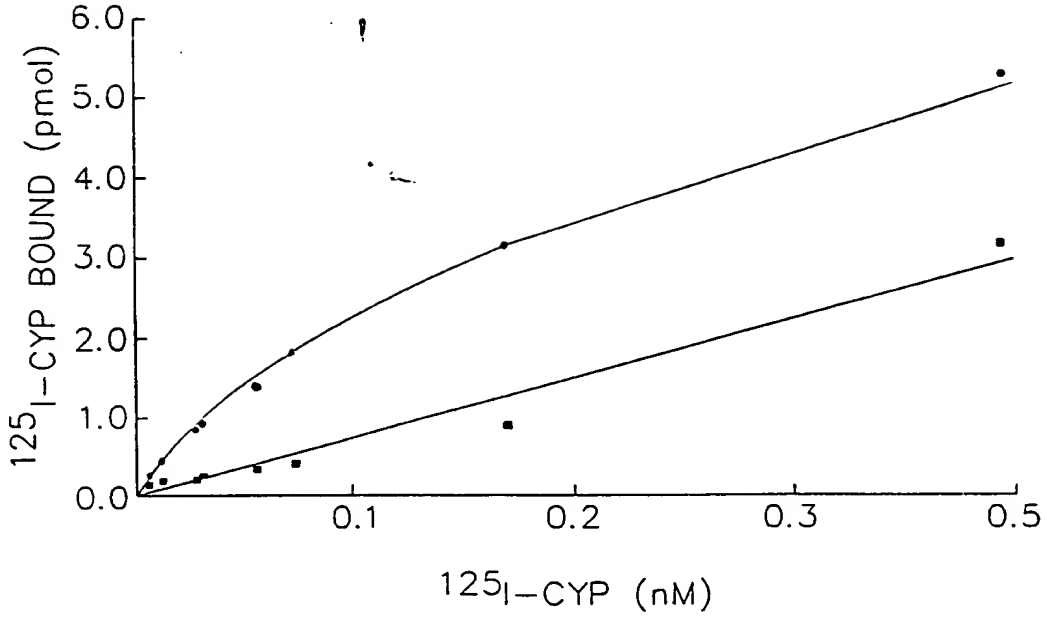
6.0
5.0
4.0
3.0
2.0
1.0
0.0
^{125}I-CYP BOUND (pmol)
0.1
0.2
0.3
0.5
^{125}I-CYP (nM)

FIG. 2A.

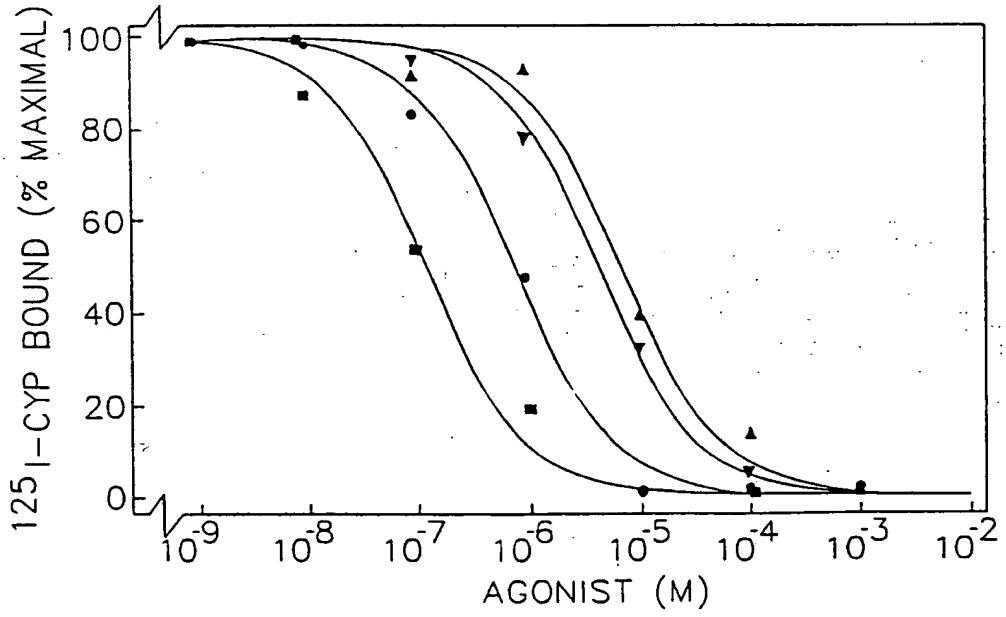
100
80
60
40
20
0
^{125}I-CYP BOUND (% MAXIMAL)
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
AGONIST (M)

FIG. 2B.

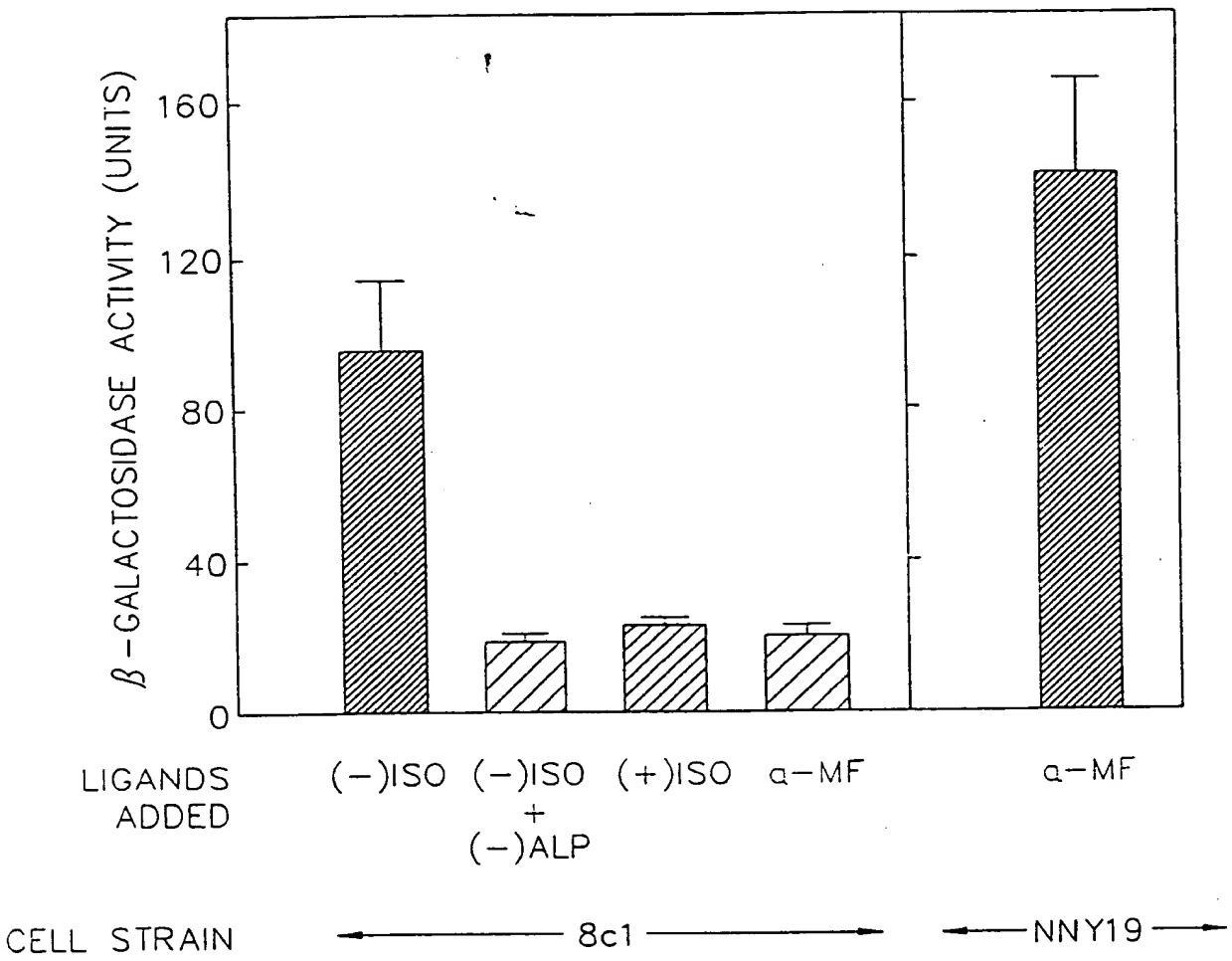
β-GALACTOSIDASE ACTIVITY (UNITS)
160
120
80
40
0
LIGANDS ADDED
(−)ISO
(−)ISO + (−)ALP
(+)ISO
a−MF
a−MF
CELL STRAIN
8c1
NNY19

FIG. 3.

WO 92/05244(1)
(C07K15/00F16B)-C07K15/00F24L8-
(C07K15/00F24L3)-(C07K15/00F24B2H)-
(C12N15/81)-[M07K203:00]-
[M07K207:00]-[M07K211:00]-
- 5- *- C07K15/00F24L8

548 165

Application Number

CH REPORT

EP 91 91 6216

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Y	FEBS LETTERS. vol. 266, no. 1-2, 18 June 1990, AMSTERDAM NL pages 21 - 25 Payette P;Gossard F;Whiteway M;Dennis M; 'Expression and pharmacological characterization of the human M1 muscarinic receptor in Saccharomyces cerevisiae.' * the whole document * ---	1-28	C12N15/12 C12N15/62 C07K13/00 C12Q1/00 G01N33/48 G01N33/566
Y	CELL vol. 50, 25 September 1987, CAMBRIDGE, NA US pages 1001 - 1010 Dietzel, C.; Kurjan, J.; 'The yeast SCG1 gene : a Galpha-like protein implicated in the a- and alpha-factor response pathway.' * page 1005, column 2, line 27 - page 1006, column 1, line 47 * ---	1-28	
Y	EP-A-0 123 544 (GENENTECH, US) 31 October 1984 * page 6, line 6 - page 7, line 9 * * Claims * ---	1-28	TECHNICAL FIELDS SEARCHED (Int. Cl.5) C12N C07K
A	EMBO JOURNAL. vol. 9, no. 3, March 1990, EYNSHAM, OXFORD GB pages 691 - 696 Nomoto S;Nakayama N;Arai K;Matsumoto K; 'Regulation of the yeast pheromone response pathway by G protein subunits.' --- -/--		

2 The supplementary search report has been drawn up for the claims attached hereto.

Place of search	Date of completion of the search	Examiner
THE HAGUE	27 MAY 1993	NAUCHE S.A.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P0401)

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
P,X	SCIENCE vol. 250, 5 October 1990, LANCASTER,PA pages 121 - 123 King K;Dohlman HG;Thorner J;Caron MG;Lefkowitz RJ; 'Control of yeast mating signal transduction by a mammalian beta 2-adrenergic receptor and Gs alpha subunit' * the whole document * -----	1-28	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)

The supplementary search report has been drawn up for the claims attached hereto.

Place of search	Date of completion of the search	Examiner
THE HAGUE	27 MAY 1993	NAUCHE S.A.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P0400)

THAT WHICH IS CLAIMED IS:

1. A transformed yeast cell containing a first heterologous DNA sequence which codes for a mammalian G protein coupled receptor and a second heterologous DNA sequence which codes for a mammalian G protein α subunit (mammalian G_α), wherein said first and second heterologous DNA sequences are capable of expression in said cell, and wherein said cell is incapable of expressing an endogenous G protein α-subunit (yeast G_α).

2. A transformed yeast cell according to claim 1, wherein said first heterologous DNA sequence is carried by a plasmid.

3. A transformed yeast cell according to claim 1, wherein said second heterologous DNA sequence is carried by a plasmid.

4. A transformed yeast cell according to claim 1, wherein said mammalian G protein α subunit is selected from the group consisting of G_s α subunits, G_i α subunits, G_o α subunits, G_z α subunits, and transducin α subunits.

5. A transformed yeast cell according to claim 1 which expresses a complex of the G protein β subunit and the G protein γ subunit ($G_{\beta\gamma}$).

6. A transformed yeast cell according to claim 5 which expresses endogenous $G_{\beta\gamma}$.

7. A transformed yeast cell according to claim 1, wherein said first heterologous DNA sequence codes for a mammalian G protein-coupled receptor selected from the group consisting of dopamine receptors, muscarinic cholinergic receptors, α-adrenergic receptors, β-adrenergic receptors, opiate receptors, cannabinoid receptors, and serotonin receptors.

8. A transformed yeast cell according to claim 1 further comprising a third heterologous DNA sequence, wherein said third heterologous DNA sequence comprises a pheromone-responsive promotor and an indicator gene positioned downstream from said pheromone-responsive promoter and operatively associated therewith.

9. A transformed yeast cell according to claim 8, wherein said pheromone responsive promoter is selected from the group consisting of the BAR1 gene promoter and the FUS1 gene promoter, and wherein said indicator gene is selected from the group consisting of the HIS3 gene and the LacZ gene.

10. A method of testing a compound for the ability to affect the rate of dissociation of G_α from $G_{\beta\gamma}$ in a cell, comprising:

providing a transformed yeast cell containing a first heterologous DNA sequence which codes for a mammalian G protein coupled receptor and a second heterologous DNA sequence which codes for a mammalian G_α, wherein said first and second heterologous DNA sequences are capable of expression in said cell, wherein said cell is incapable of expressing endogenous G_α, and wherein said cell expresses $G_{\beta\gamma}$;

contacting said compound to said cell; and

detecting the rate of dissociation of G_α from $G_{\beta\gamma}$ in said cell.

11. A method according to claim 10, wherein said yeast cells are provided in an aqueous solution and said contacting step is carried out by adding said compound to said aqueous solution.

12. A method according to claim 10, wherein said mammalian G protein α subunit is selected from the group consisting of G_s α subunits, G_i α subunits; G_o α subunits, G_z α subunits, and transducin α subunits.

13. A method according to claim 10, wherein said yeast cell expresses endogenous $G_{\beta\gamma}$.

14. A method according to claim 10, wherein said first heterologous DNA sequence codes for a mammalian G protein-coupled receptor selected from the group consisting of dopamine receptors, muscarinic cholinergic receptors, α-adrenergic receptors, β-adrenergic receptors, opiate receptors, cannabinoid receptors, and serotonin receptors.

15. A method according to claim 10, said yeast cell further comprising a third heterologous DNA sequence, wherein said third heterologous DNA sequence comprises a pheromone-responsive promotor and an indicator gene positioned downstream from said pheromone-responsive promoter and operatively associated therewith;

and wherein said detecting step is carried out by monitoring the expression of said indicator gene in said cell.

16. A DNA expression vector capable of expressing a transmembrane protein into the cell membrane of yeast cells, comprising:

a first segment comprising at least a fragment of the extreme amino-terminal coding sequence of a yeast G protein coupled receptor; and

a second segment downstream from said first segment and in correct reading frame therewith, said second segment comprising a DNA sequence encoding a heterologous G protein coupled receptor.

17. A DNA expression vector according to claim 16, wherein a fragment of the extreme amino-terminal coding sequence of said heterologous G protein coupled receptor is absent.

18. A DNA expression vector according to claim 16, wherein said first and second segments are operatively associated with a promoter operative in a yeast cell.

19. A DNA expression vector according to claim 18, wherein said promoter is the GAL1 promoter.

20. A DNA expression vector according to claim 16, wherein said first segment comprises at least a fragment of the extreme amino-terminal coding sequence of a yeast pheromone receptor.

21. A DNA expression vector according to claim 16, wherein said first segment comprises at least a fragment of the extreme amino-terminal coding sequence of a yeast pheromone receptor selected from the group consisting of the STE2 gene and the STE3 gene.

22. A DNA expression vector according to claim 16, further comprising at least a fragment of the 5'-untranslated region of a yeast G protein coupled receptor gene positioned upstream from said first segment and operatively associated therewith.

23. A DNA expression vector according to claim 16, further comprising at least a fragment of the 5'-untranslated region of a yeast pheromone receptor gene positioned upstream from said first segment and operatively associated therewith.

24. A DNA expression vector according to claim 23, wherein said yeast pheromone receptor gene is selected from the group consisting of the STE2 gene and the STE3 gene.

25. A DNA expression vector according to claim 16, said vector comprising a plasmid.

26. A DNA expression vector according to claim 16, said second segment comprising a DNA sequence encoding a mammalian G protein coupled receptor.

27. A DNA expression vector according to claim 16, said second segment comprising a DNA sequence encoding a mammalian G protein-coupled receptor selected from the group consisting of dopamine receptors, muscarinic cholinergic receptors, α-adrenergic receptors, β-adrenergic receptors, opiate receptors, cannabinoid receptors, and serotonin receptors.

28. A yeast cell carrying a DNA expression vector according to claim 16.

EXAM

I 152 OXYTOCIN-INDUCED OSCILLATIONS IN INTRACELLULAR CALCIUM IN DEPOLARISED CULTURED HUMAN MYOMETRIAL SMOOTH MUSCLE CELLS. Joanna M. Morgan, Stephen Lynn, James I. Gillespie and John R. Greenwell, Department of Physiological Sciences, The Medical School, University of Newcastle upon Tyne, NE2 4HH. U.K.

Spontaneous and agonist-induced oscillations in intracellular calcium have been recorded in cultured human myometrial smooth muscle cells. These oscillations persist in solutions containing high concentrations of potassium (140mM) suggesting that the underlying mechanism does not involve the cyclical operation of voltage-dependant ion channels. Intermediate increases in external potassium (10-70mM) can lead to an increase in the frequency of the calcium oscillations suggesting that membrane potential regulated mechanisms do operate in these cells but that their function may be primarily to modulate the frequency of the calcium oscillations.

The hypothesis is proposed that repetitive calcium spikes involve the cyclical release of calcium from an internal store, possibly a single calcium store, sensitive to inositol trisphosphate (IP_3) and activated by intracellular calcium. As a result of membrane depolarisation an increased calcium influx could modulate the frequency of the calcium spikes by influencing basal levels of cytoplasmic calcium. This cyclical release may be modulated by elevating IP_3 (via agonist stimulation) which functions effectively as a modulator rather than a primary intracellular signal to alter the sensitivity of the calcium release process to calcium.

In human labour the plasma concentration of oxytocin and prostaglandins do not change in the period leading up to contractions and do not fluctuate during the early phases of labour. Therefore, it is not clear what contribution these hormones play in the phasic activity of the human myometrium. However, if the role of such hormones was to raise IP_3 to modulate the cyclical release of calcium from internal stores, possibly triggered or augmented by a calcium influx via voltage gated calcium channels, the myometrium would function in a phasic manner in the presence of a steady background level of hormone.

I 153 AUTOCRINE STIMULATION OF YEAST THROUGH HUMAN G-COUPLED RECEPTORS. Murphy, A.J.M.; Paul J.; Manfredi, J.; Silverman, L.; Trueheart, J.; McKinney, M. and Broach, J. Cadus Pharmaceutical Corporation, 180 Varick St., N.Y., N.Y.

Response to mating factors in the yeast *Saccharomyces cerevisiae* is coupled to transcription through a seven transmembrane receptor, a heterotrimeric G-protein and a series of protein kinases. By incorporating a pheromone responsive selectable marker construct (fus1-HIS3), we have generated a growth read-out for activation of the pathway. Through a series of additional modifications, including the replacement of the yeast Gα subunit (GPA1) with a modified human $G\alpha_{i2}$, we have functionally substituted the yeast pheromone receptor with the human angiotensin II-type I receptor. When expressed in yeast, this receptor binds ligands with appropriate specificity and affinity. Co-expression of the angiotensin II octapeptide generates an autocrine loop in which the endogenously produced peptide stimulates the co-expressed receptor. This yields prototrophic growth dependent on expression of both angiotensin receptor and the angiotensin II peptide. This system provides a facile microbiological assay for angiotensin II variants, receptor mutants and external agents that affect signal transduction through this mammalian G-coupled receptor.

I 154 A DOMINANT NEGATIVE MUTANT OF G_{i2} INHIBITS PHOSPHOLIPASE A_2 STIMULATION IN CHO CELLS. Rosemary Murray-Whelan, John Reid*, Isabelle Pluz and Werner Schlegel, Fondation pour Recherches Medicales, University of Geneva, 64 Av de la Roseraie, 1211 Geneva 4 and *Glaxo IMB, 64 Chemin des Aulx, 1228 Plan-les-Ouates, Geneva, Switzerland.

The specificity of interaction of receptors, G proteins and effectors is a central question in cell regulation. A powerful approach to this question is the use of mutated G proteins which either interrupt or constitutively activate the signalling pathway. We have generated a dominant inhibitor of G_{i2} which demonstrates the role of G_{i2} in PLA_2 activation in CHO cells. A mutant form of α_{i2} in which glycine 203 was converted to threonine (G203T) was made by site-directed mutagenesis. When expressed in CHO cells, G203T demonstrates a dominant negative phenotype. G203T had no effect on basal levels of arachidonic acid release. However, both thrombin and ATP stimulation of PLA_2 were 50-60% inhibited by G203T. In contrast, stimulation of calcium release by either thrombin or ATP, which is pertussis toxin insensitive, was not affected by the mutant. The effect of the mutant is thus specific for G_{i2} mediated signal transduction. The *gip* oncogene (α_{i2} Q205L mutant) is a constitutively active mutant of G_{i2} which has previously been shown to inhibit both adenylate cyclase and PLA_2 in CHO cells. Unlike Q205L, however, G203T had no effect on stimulation of adenylyl cyclase activity by either cholera toxin or forskolin. In addition, receptor-independent activation of arachidonic acid release by PMA was unaffected by G203T, while PLA_2 activation by PMA is decreased by 20-25% in Q205L-expressing cells. These data indicate that the inhibitory effect of G203T on thrombin and ATP stimulation of PLA_2 is mediated directly at the G protein level, while Q205L inhibition is probably secondary to activation and subsequent desensitization. Thus mutation of glycine 203 of the α_{i2} protein inactivates G_{i2} and produces a dominant negative inhibitor of endogenous G_{i2} activation of PLA_2.

I 155 CALCIUM RESPONSES TO TRH: AGONIST-STIMULATED EFFLUX OF CYTOPLASMIC CALCIUM. Eric J. Nelson and Patricia M. Hinkle, Dept. of Pharmacology, Univ. of Rochester Med. Sch., Rochester, NY 14642

TRH is a calcium-mobilizing hormone that stimulates immediate release of Ca^{2+} from intracellular, IP_3-sensitive stores. Using single cell imaging we have investigated the TRH response in Fura-2-loaded GH_3 pituitary cells expressing endogenous receptor and Hela cells transfected with the TRH receptor cDNA. The amplitude of the initial $[Ca^{2+}]i$ transient increased as the TRH concentration was raised. However, the duration of the initial $[Ca^{2+}]i$ transient decreased at high TRH concentrations, suggesting that TRH was stimulating both increased release of Ca^{2+} from the endoplasmic reticulum via IP_3 and increased clearing of Ca^{2+} from the cytoplasm. TRH caused $[Ca^{2+}]i$ to go down when it was added to GH_3 cells after $[Ca^{2+}]i$ had been increased by: ionomycin, a calcium ionophore; high potassium, which depolarizes cells and increases influx through L-channels; or thapsigargin, which depletes IP_3-sensitive calcium stores. TRH-stimulated Ca^{2+} clearing could not be mimicked by phorbol esters, suggesting that protein kinase C activation is not sufficient to cause the effect. TRH-induced Ca^{2+} clearing was dose-dependent in both cell lines. Following depletion of IP_3-sensitive Ca^{2+} pools, TRH caused Ca^{2+} clearing without the TRH-evoked transient increase in $[Ca^{2+}]i$. TRH-stimulated Ca^{2+} clearing did not result from an effect on calcium channels because: TRH increased Ca^{2+} clearing in transfected Hela cells, which lack calcium channels; in GH_3 cells, TRH increased Ca^{2+} clearing while L-type calcium channels were blocked; finally, TRH increased Ca^{2+} clearing when GH_3 cells were incubated in EGTA to chelate extracellular Ca^{2+} or in medium containing a 100-fold range of Ca^{2+} concentrations. These results show that TRH causes $[Ca^{2+}]i$ to fall by stimulating Ca^{2+} efflux. Complete replacement of extracellular sodium did not inhibit TRH-stimulated Ca^{2+} clearing, so Ca^{2+} efflux via the sodium-calcium exchanger is not involved. The results suggest that calcium-mobilizing agonists may activate a plasma membrane calcium ATPase. In summary, TRH initiates both a $[Ca^{2+}]i$ transient and agonist-activated clearing of cytosolic Ca^{2+}, which prevents prolonged elevation of $[Ca^{2+}]i$ and terminates the $[Ca^{2+}]i$ signal.

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(71) Applicant: DUKE UNIVERSITY [US/US]; Erwin Road, Durham, NC 27706 (US).

(72) Inventors: KING, Klim ; 3925 Linden Terrace, Durham, NC 27705 (US). DOHLMAN, Henrik, G. ; 2226 Stewart Street, Berkeley, CA 94705 (US). CARON, Marc. G. ; 2606 Evans Drive, Durham, NC 27705 (US). LEFKOWITZ, Robert, J. ; 3539 Hamstead Court, Durham, NC 27707 (US).

(74) Agents: SIBLEY, Kenneth, D. et al.; Bell, Seltzer, Park & Gibson, P.O. Drawer 34009, Charlotte, NC 28234 (US).

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(54) Title: EXPRESSION OF G PROTEIN COUPLED RECEPTORS IN YEAST

(57) Abstract

Disclosed is a transformed yeast cell containing a first heterologous DNA sequence which codes for a mammalian G protein coupled receptor and a second heterologous DNA sequence which codes for a mammalian G protein α subunit (mammalian G_α). The first and second heterologous DNA sequences are capable of expression in the cell, but the cell is incapable of expressing an endogenous G protein α-subnit (yeast G_α). The cells are useful for screening compounds which affect the rate of dissociation of G_α from $G_{\beta\gamma}$ in a cell. Also disclosed is a novel DNA expression vector useful for making cells as described above. The vector contains a first segment comprising at least a fragment of the extreme amino-terminal coding sequence of a yeast G protein coupled receptor. A second segment is positioned downstream from the first segment (and in correct reading frame therewith), with the second segment comprising a DNA sequence encoding a heterologous G protein coupled receptor.

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